American Journal of Hematology 59:279–287 (1998)

Role of Caspases 1 and 3 and Bcl-2-Related Molecules in Endothelial Cell Apoptosis Associated With Thrombotic Microangiopathies

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We have defined an in vitro model for the study of microvascular endothelial cell (EC) apoptosis mediated by plasma from patients with various forms of thrombotic thrombocytopenic purpura (TTP) and hemolytic-uremic syndrome (HUS). This system reproduces a variety of histopathologic and ultrastructural features of tissue EC involved in TTP/sporadic HUS, suggesting that apoptotic EC injury is a primary pathophysiologic event in the thrombotic microangiopathies. We now document the ability of tetrapeptide-based inhibitors of interleukin 1β-converting enzyme (ICE)-like caspase 1 and cysteine protease protein (CPP)-32-like caspase 3, two members of a novel class of cysteine proteases involved in final pathways to apoptosis, to block TTP/sporadic HUS plasma-mediated apoptosis. Overexpression of Bcl-X_L via gene transfer suppressed this apoptosis by 70%. Transduction of EC with the Bcl-2 homolog A1 had a more limited protective effect. These findings support a role for apoptosis-linked cysteine proteases in the pathophysiology of TTP and sporadic HUS, and raise the possibility that specific apoptosis inhibitors may have a role in the experimental therapeutics of these syndromes. Am. J. Hematol. 59:279–287, 1998. © 1998 Wiley-Liss, Inc.

Key words: caspase; cell death, programmed; hemolytic-uremic syndrome; human immunodeficiency virus; protease inhibitors; purpura, thrombotic thrombocytopenic

INTRODUCTION

Endothelial cells (ECs) are particularly sensitive to disruption of homeostatic mechanisms controlling cell proliferation and survival, given their prolonged in vivo doubling times, measured in months to years [1]. Indeed, accelerated EC apoptosis appears to play a critical role in many vascular pathologies [2]. We [3–5] have been exploring the role of apoptosis in thrombocytopenic purpura (TTP) and adult/sporadic hemolytic-uremic syndrome (HUS), two closely related disorders characterized by idiopathic microangiopathy [6]. We reported that TTP and sporadic HUS plasmas induce apoptosis in primary human microvascular (MV) ECs of renal, cerebral, and dermal origin, but not in large vessel ECs or MVECs of pulmonary lineage [3–5]. This reflects the pathology and distribution of platelet thrombi in both disorders. Similar changes were not seen with plasmas from patients with disseminated intravascular coagulation or idiopathic thrombocytopenic purpura, some of whom had been multiply transfused [3–5].

These observations have now been replicated in another laboratory [7]. The in vitro changes parallel the high frequency of apoptotic cells recognized in glomerular capillary lumina, presumably of MVEC origin, in adult HUS [8], and the finding of apoptotic nuclei in the microvasculature of involved tissues from TTP patients, documented by morphology and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay [Dang C, Magid M, Weksler B, Chadburn A, Laurence J; submitted]. We now sought to de-

Grant sponsor: National Institutes of Health; Grant numbers: HL55646, AI41327, and DE11348 (to J.L.).

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Received for publication 5 March 1998; Accepted 12 August 1998

termine mechanisms for this apoptosis, and to define potential interventions.

Apoptosis may be divided into initiation, effector, and degradation phases. Although the initiation stage depends upon the nature of the trigger—yet unknown for TTP/sporadic HUS in vivo or in our MVEC model—the effector and cell degradation phases are common to many apoptotic processes [9]. The interleukin 1β-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases, members of a class of cysteine proteases known as caspases (cysteinyl aspartate-specific proteinases), play a crucial role in programmed cell death [10,11]. Activated caspases are organized into protease cascades, with different pathways activated by divergent stimuli even in the same cell [12]. Caspase 1 (ICE-like) and caspase 3 (CPP32-like) are up-regulated in TNF-α and Fas (CD95)-triggered apoptosis [13]. TNF-α was not involved in TTP/HUS plasma-mediated EC death in our system, but Fas induction was a predominant feature of this model [3–5]. In addition, TTP plasmas contain elevated levels of cysteine-like proteases [14], and aurintricarboxylic acid, a nonspecific protease inhibitor [15], blocked TTP plasma-mediated MVEC apoptosis in vitro [3]. We thus sought to examine the role of caspases 1 and 3, and two apoptotic regulatory factors of the Bcl-2 family found in ECs, A1, and Bcl-X_L, in TTP/sporadic HUS-associated MVEC apoptosis.

MATERIALS AND METHODS

Patients

Plasma samples were obtained from heparinized or ethylenediaminetetraacetic acid (EDTA)-treated venous blood of six human immunodeficiency virus (HIV) seronegative asymptomatic controls, five HIV seronegative, and one HIV seropositive adult patients with TTP, and one HIV seronegative adult with sporadic HUS. Four experimental samples used repeatedly in this report are coded as: TTP-1 and TTP-4, female, HIV seronegative, TTP; TTP-2, female, HIV seronegative, sporadic HUS; and TTP-3, female, HIV+, TTP.

TTP was diagnosed according to the following criteria: fever, unexplained oral temperature >38.0°C; neurologic dysfunction; any new abnormality on general medical neuropsychiatric exam; renal dysfunction, serum creatinine ⩾1.2 mg/dl, or >150% of previous baseline; and thrombocytopenia, platelet count <150,000/mm^3. Sporadic HUS was diagnosed by: thrombocytopenia, platelet count <150,000/mm^3; renal dysfunction, serum creatinine ⩾1.5 mg/dl; and the absence of clinical neurologic disease. All patients had microangiopathy on peripheral blood smear.

Endothelial Cell Cultures

Four primary human MVECs were utilized: MVEC-1 (HMVEC 2753, dermal origin; Clonetics, San Diego, CA); HMVEC-G (renal glomerular; Cell Systems, Kirkland, WA); HMVEC-B (cerebral; Cell Systems); and TEC (tonsil; Cell Systems). ECs were maintained in T-25 flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in modified MCDB 131 medium containing recombinant human epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), bovine brain extract, heparin (10 μg/ml), amphotericin B (50 ng/ml), gentamicin (50 μg/ml), 10% fetal bovine serum (FBS), and 10% pooled human serum, and used in passages 2 to 7. All cells were positive for expression of von Willebrand factor and CD36, and had a doubling time of approximately 36 hr. Subcultures involved a 5–10 min exposure to 0.025% trypsin/0.01% EDTA or 0.1% collagenase, followed by washing with phosphate buffered saline (PBS), pH 7.2. To insure some uniformity of culture conditions, experiments were performed in ''apoptosis culture medium'' devoid of human serum: medium 199 plus 5% FBS, epidermal growth factor, heparin, amphotericin B and gentamicin, following a 4–6 hr preincubation of ECs in that medium.

Apoptosis Assays

These assays were described previously [3–5]. Briefly, ECs were washed with PBS, assessed for viability by trypan blue dye exclusion, then plated in chambers of 12 well plates, coated with 0.2% gelatin in water, at 0.15×10^6 viable cells/ml in apoptosis culture medium, alone or with dilutions of various plasmas. Cells were harvested 18 hr later, fixed in 70% cold ethanol, incubated for 20 min at 4°C with propidium iodide (50 μg/ml) in the presence of RNaseA (300 U/ml), and 5×10^3 cells analyzed in the cytofluorograph (EPICS Elite, Coulter, Hialeah, FL).

Apoptosis was recognized or quantitated in viable cells by flow cytometry involving computer-assisted DNA histogram analysis of propidium iodide-labelled cells with calculation of pre-G_1 A_0 peaks, defined by computer software (MCycle Av; Phoenix Flow Systems, San Diego, CA) [4]. A_0 is fit as a normally distributed curve of hypodiploid DNA just prior to the narrow diploid DNA area. Some experiments involved two separate determinations, with A_0 values for a given plasma sample and cell type of similar passage number varying by ⩽15%.

Expression of Apoptosis-Associated Transcripts

Fas has a central role in the regulation of apoptosis in diverse cell types via interactions with its ligand, FasL [16]. We had documented a close association between TTP/sporadic HUS plasmas capable of inducing MVEC

TABLE I. Reverse Transcription-Polymerase Chain Reaction Primer Pairs for Apoptosis-Associated Transcripts

Molecule	Oligomer primer pairs (5′-sense/5′-antisense)	Position	Amplicon size	Reference
Fas (CD95)	CAAGTGACTGACATCAACTCC CCTTGGTTTTCCTTTCTGTGC	271–820	549	17,18
Caspase 1 (ICE)	ACCTTAATATGCAAGACTCTCAAGGAG GCGGCTTGACTTGTCCATTATTGGATA	301–490	189	19
Caspase 3 (CPP-32)	TGGAAGCGAATCAATGGACTCTGG GCATACAAGAAGTCGGCATCCACT	290–811	521	20
Bcl-2	GTGCCACCTGTGGTCCACCTG CTTGTGGCTCAGATAGGCACC	264–718	454	2
A1	ACCAGGCAGAAGATGACAGACTGT GTCCTTTCTGGTCAACAGTATTGC	67–617	550	2
Bcl-X_L	CGACGAGTTTGAACTGCGGTA GTGTCTGGTCATTTCCGACTG	416–844	428	21
β-actin	TGACGGGGTCACCCACACTGTGCCCATCTA CTAGAAGCATTTGCGGTGGACGATGGAGGG		661	Stratagene, Inc.

apoptosis and up-regulation of Fas mRNAs in those cells [4]. Expression of Fas transcripts was initially evaluated by two techniques: quantitative reverse transcription-polymerase chain reaction (RT-PCR), using PCR Mimic (Clontech, Palo Alto, CA) technology [17] and a non-quantitative RT-PCR [3]. Parallel results were obtained, and thus only the latter technique was performed here.

Total cellular RNAs were isolated from 1×10^6 cells/sample by the TriZOL (GIBCO-BRL, Gaithersburg, MD) method [3]. RNAs were treated with RNase-free DNase, and a constant amount (1 μg) of RNA was reverse transcribed into cDNA using 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). cDNA aliquots of equal volume were then amplified by PCR, as described [3]. Reaction products were run on a 1.4% agarose gel, visualized by ethidium bromide staining under ultraviolet illumination, and were photographed. Integrity of initial RNAs was checked by amplifying β-actin. Relative levels of product were estimated by densitometry scanning of gel photographs [17]. All RT-PCR oligomer sequences [2,17–21] and amplicon sizes are listed in Table I.

Immunoblotting

Caspase expression in various MVEC populations was evaluated by immunoblotting. HMVEC-G cells (1×10^6/condition) were exposed to normal or TTP plasmas overnight, then were lysed in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodiu, deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride [PMSF], and 50 mM Tris, pH 8.0). Fifty μg of protein lysate, quantitated by bicinchoninic acid (BCA) assay (Pierce) were separated by SDS/PAGE. Proteins were transferred to PVDF membrane using an LKB transfer apparatus and probed with a monoclonal antibody to CPP32 (Oncogene Research, Cambridge, MA) or rabbit polyclonal antibody to ICE (Oncogene Research). The secondary antibody was either an horseradish peroxidase (HRP)-goat anti-mouse immunoglobulin (Ig)G or an HRP-goat anti-rabbit IgG (KP Laboratories). Detection involved enhanced chemiluminescene, performed according to the manufacturer's directions (Amersham).

Anti-Apoptosis Reagents

The trisodium salt of aurintricarboxylic acid (ATA) (Sigma, St. Louis, MO) was dissolved in PBS, pH 7.4, and used at final concentrations of 0.01–1 μM. Okadaic acid (Gibco-BRL) was prepared as a 1 mM stock in water, and kept at −20°C. The tetrapeptide-based cysteine protease inhibitors DEVD-H (Ac-Asp-Glu-Val-aspartic acid aldehyde), YVAD-H (Ac-Tyr-Val-Ala-aspartic acid aldehyde), and YVAD-CMK (Ac-Tyr-Val-Ala-Asp-chloromethylketone) were purchased from Bachem Bioscience (King of Prussia, PA). DEVD-H was prepared as a 100 μM stock in culture medium, and YVAD-H as a 50 μM stock in water, and kept at −20°C. YVAD-CMK was dissolved in dimethylsulfoxide (DMSO) to 20 μM and kept at −20°C. An assessment of the degree of specificity and the activity of these compounds for intact cells in vitro [15,22–25] and in vivo [22,26] are provided in Table II.

Gene Transfer of Bcl-2 Homoloques

A FLAG octapeptide N-terminal tagged human A1 gene was generated by PCR and ligated into the HindIII/HpaI site of the replication-deficient retroviral vector pLNCX, as described [27]. The coding region of Bcl-X_L DNA was similarly ligated into the HindII/HpaI site of pLNCX. The viral long terminal repeat drives expression of neomycin resistance (neo^R), whereas a cytomegalovirus promoter drives gene expression. Preparation of packaging cell lines and vector containing supernatants from transduced amphotropic PA317 cells was previously detailed [27]. One milliliter of supernatants from PA317 cells containing neo^R, FLAG-A1-neo^R, or FLAG-

TABLE II. Caspase and Protease Inhibitors Used

Compound	Molecular weight	Primary target	Specificity for ICE superfamily	IC_{50} (μM)[a]	Reference
YVAD-H	492.5	Caspase 1	High	0.7–2.5	22
YVAD-CMK	541	Caspase 1	High	1–10	22,23
DEVD-H	502.5	Caspase 3	Low	50–100	22
Okadaic acid	805	Serine/threonine proteases	None	0.1–1	24
Aurintricarboxylic acid (monomer)	422	Proteases and endonucleases	None	0.01–1	15,25

*ICE, interleukin 1β-converting enzyme.
[a]Citations provided for use with intact cells in vitro. In vivo activities for the first three compounds are described in reference 26.

Bcl-X_L-neo^R was used to transduce 0.5×10^6 MVECs of dermal, renal, and cerebral origin, plated to 60–80% confluency in wells of six chamber macrowell plates previously coated with 0.2% gelatin in water. Transduced cells were selected and expanded over 10–14 days of culture in 150 μg/ml G418 (Gibco-BRL). This concentration of G418 was toxic only to MVEC lacking the neo^R gene.

Cloning of transduced cells was not attempted, avoiding artifacts secondary to retroviral integration. The efficiency of A1 and Bcl-X_L gene expression was estimated by staining of both untreated cells and cells permeabilized with 4% paraformaldehyde with an anti-FLAG mouse monoclonal antibody (mAb) (Scientific Imaging Systems, Rochester, NY), followed by an FITC conjugated goat anti-mouse IgG (TAGO) and flow cytometric analysis. Results were checked by RT-PCR for A1 and Bcl-X_L, using techniques described above, and primers outlined in Table I.

Various transduced cell populations were then challenged with control or TTP plasmas. A positive control for A1 and Bcl-X_L activity consisted of EC exposure to TNF-α (0.05 ng/ml; Sigma) in the presence of actinomycin D (1 μg/ml; Sigma).

RESULTS

Apoptosis Data

We first examined the baseline DNA histogram patterns of RNase-treated and propidium iodide stained primary human MVECs of dermal, renal, cerebral, and oral mucosal (tonsil) origin. Cells suspended in apoptosis culture medium were plated in gelatin-coated macrowells, then exposed for 18 hr to buffer, control plasmas, or plasmas derived from patients with TTP and sporadic HUS. MVECs cultured in the presence of buffer or 1% normal plasma typically showed similar cell-cycle profiles, with low percentages of cells in A_0. Representative baselines for tonsil ($A_0 = 0$) and dermal ($A_0 = 2.1\%$) cells in control plasmas are provided in Figure 1. Exposure of all MVECs tested to ⩾1:100 dilutions of TTP/sporadic HUS plasmas gave classic apoptotic patterns, as illustrated for three TTP plasmas in Figure 1, with A_0 values of 12.1 to 52.5%. This is consistent with results with TTP and sporadic HUS plasmas in MVECs of dermal, renal, and cerebral origin from our lab and others [3–5,7], and extends the observation to tonsil MVECs.

Expression of Apoptosis-Associated Transcripts and Proteins

Fas transcripts were induced by plasma from patients with TTP and sporadic HUS in MVECs of dermal and renal (Fig. 2) and cerebral and tonsillar (not shown) origins. Expression of mRNA for caspase 1 and caspase 3 was then investigated by RT-PCR in dermal (MVEC-1) and renal (HMVEC-G) cells. Baseline expression of both caspases was at the limits of detectability in HMVEC-G, and of low level in MVEC-1 (Fig. 2). Eighteen hours after exposure to plasmas from all three patients tested, one HIV− and HIV+ patient with TTP, and one HIV− individual with adult HUS, increases in caspase 3 mRNA of 3–5-fold, and caspase 1 of 1.5–3-fold were noted for HMVEC-G (summary of three experiments, with one representative series in Figure 2). For MVEC-1, a 1.5–2-fold increase in caspases 1 and 3 mRNAs was noted with two of the three plasmas (Fig. 2). Time-course data showed that maximal expression of mRNAs for both proteases occurred 12–24 hr after exposure to TTP or sporadic HUS plasmas. Albeit minor in magnitude, these changes parallel up-regulation of caspase transcripts seen following Fas cross-linking as well as certain other apoptotic stimuli [23,25].

Consistent with these relatively minor alterations in caspase mRNAs, there was no detectable alteration in caspase 1 (ICE) protein levels, and only a 1.5-fold increase in caspase 3 (CPP-32) protein by immunoblotting (data not shown).

The physiologic relevance of these changes was then sought. Suppression of new protein synthesis by addition of cycloheximide (50 μg/ml) concomitantly with exposure to control or TTP plasmas had no effect on the extent of apoptosis (Fig. 1, middle panel). Thus, translation of new caspase mRNAs was not essential to cell death in this system.

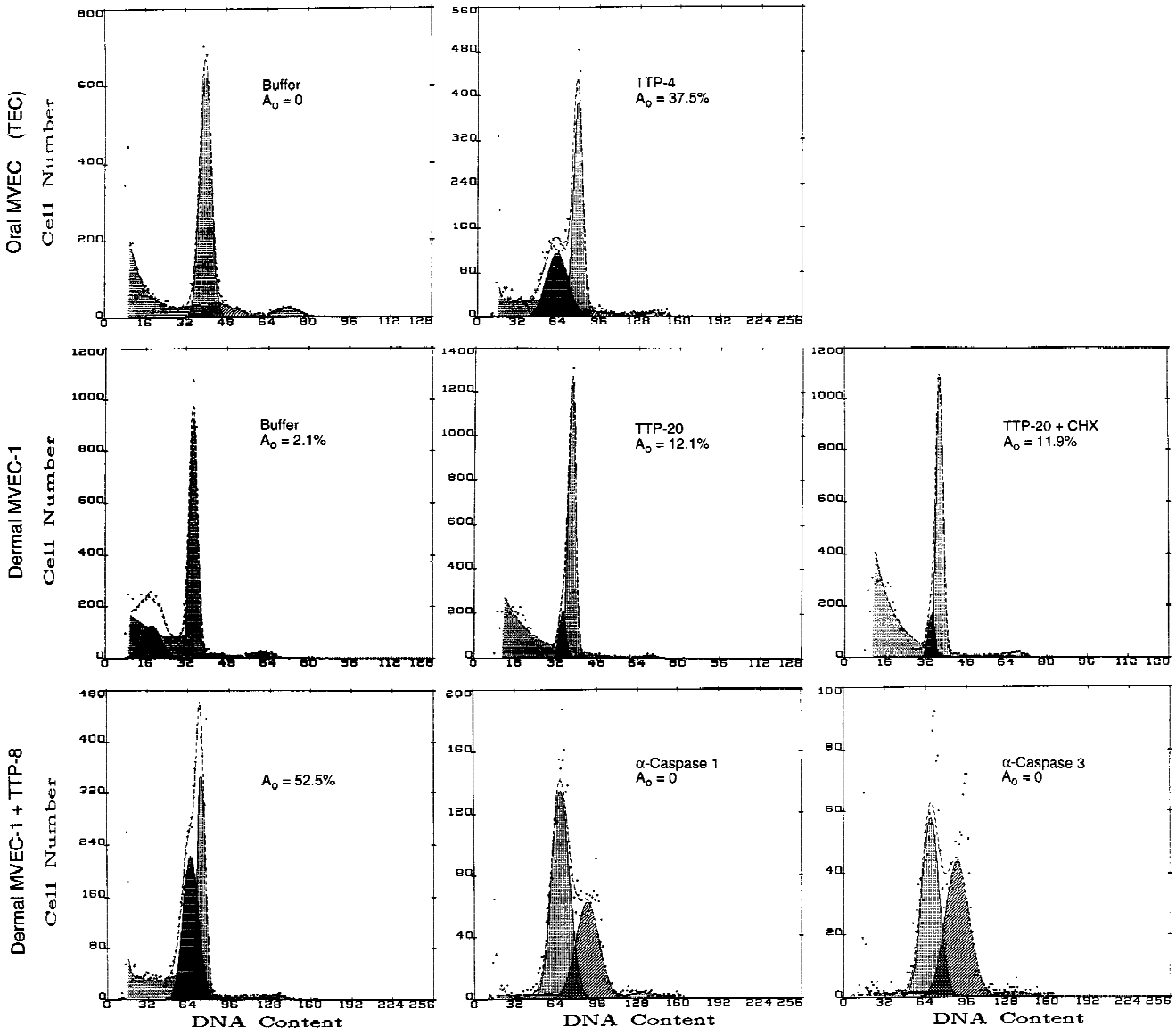

Fig. 1. DNA histograms of MVECs exposed to normal and TTP plasmas, and the impact of cysteine protease inhibitors. Primary human microvascular ECs from various tissues were cultured for 18 hr in apoptosis culture medium in the presence of a 1:100 dilution of plasma from control donors or TTP patients. DNA histograms of ethanol-fixed, RNase treated and propidium iodide-stained cells were obtained and analyzed by Phoenix Flow Systems computer software, as described in the text. A_0 peak values are indicated within each histogram, representing the more heavily shaded region just to the left of the large G_1/S peak. In select cultures either cycloheximide (CHX) (50 μg/ml) or caspase inhibitors (see Table II for specificities) were added concomitantly with plasma. Top panel: oral mucosal (tonsil) EC exposed to buffer or TTP-4 plasma; middle panel: dermal (MVEC-1) exposed to buffer, TTP-20 plasma, or TTP-20 plasma plus CHX; and lower panel: MVEC-1 exposed to TTP-8 plasma in the presence of buffer, 10 μM of the caspase 1 inhibitor YVAD-H, or 100 μM of the caspase 3 inhibitor DEVD-H.

Expression of Potential Anti-Apoptotic Factors

We extended reports that transformed ECs lack Bcl-2 [2] to our primary MVECs, using RT-PCR assays and primers listed in Table I. No Bcl-2 mRNA was seen in cells cultured with either normal or TTP plasma (data not shown). We then sought evidence for transcription of the Bcl-2 homolog A1 and of Bcl-X_L in our cells. A1 is normally up-regulated by TNF-α, IL-1, and protein kinase inducers such as phorbol esters, but is unaffected by vascular growth factors [2]. Bcl-X_L is constitutively expressed in ECs and is not induced by TNF-α [27]. Dermal and renal MVECs were exposed to normal or TTP plasmas (1:100 dilution) for 18 hr, and RNAs isolated. Transcripts for A1 and Bcl-X_L showed no change following exposure to TTP plasmas.

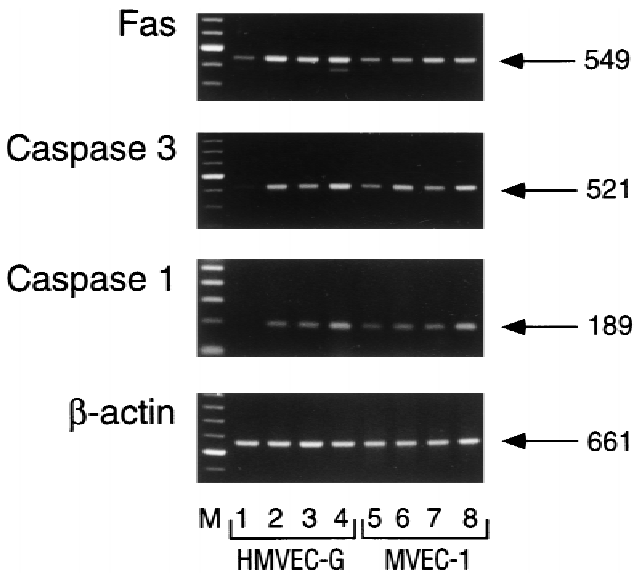

Fig. 2. RT-PCR for expression of apoptosis-related molecules in endothelial cells. PCR analysis of cDNA reverse transcribed from RNAs obtained from microvascular ECs of renal (HMVEC-G) and dermal (MVEC-1) origins, cultured for 18 hr in the presence of various plasmas (1:100 dilutions), was performed. Plasma sources are described in the text. Primers for Fas, caspase 1, caspase 3 and β-actin (a control for integrity of the starting materials) are given in Table I. The two bands for Fas recognized in some lanes represent the membrane-bound and secreted forms of this molecule. Lane 1, renal MVEC + normal plasma; lane 2, renal MVEC + TTP-2; lane 3, renal MVEC + TTP-1; lane 4, renal MVEC + TTP-3; lane 5, dermal MVEC + normal plasma; lane 6, dermal MVEC + TTP-2; lane 7, dermal MVEC + TTP-1; and lane 8, dermal MVEC + TTP-3.

Suppression of TTP/HUS Plasma-Associated MVEC Apoptosis by Caspase Inhibitors

We sought to determine whether caspase 1 and/or caspase 3 activity was involved in TTP/HUS plasma-mediated EC apoptosis. First, nonspecific agents, including okadaic acid, an inhibitor of serine/threonine proteases, and ATA, a general protease inhibitor, were evaluated. ATA blocked TTP/HUS plasma-mediated EC apoptosis by >75% in all MVECs tested (Table III and and data not shown). Okadaic acid, at the relatively high concentration of 0.5 μM, had a similar effect (Table III). Next, tetrapeptides of varying selectivity for the ICE superfamily of apoptosis-associated molecules (Table II) were utilized. DEVD-H, which has poor cellular uptake [22], had no impact on TTP plasma-mediated apoptosis at doses up to 50 μM (not shown); inhibition was seen only at the highest dose tested (100 μM). However, complete suppression of hypodiploid A_0 peaks was obtained with ⩾10 μM YVAD-H (Table III). Dose-response experiments with YVAD-H (Table III) gave IC_{50}s consistent with that recorded for other in vitro systems [24,26].

Effects of Overexpression of A1 and Bcl-X_L on Apoptosis

Dermal, renal, and cerebral MVECs were transduced with retroviral constructs of neo^R-linked and FLAG-tagged A1 and Bcl-X_L, then selected in G418. Control cells were transduced with the neo^R-containing vector, LNCX. G418 was excluded from the culture medium for 48 hr prior to an experiment, and during the course of the 18 hr plasma exposure. Three separate transductions and neo^R selections were performed for cerebral and dermal MVECs, and one for renal MVECs. Transgene expression was assessed by flow cytometry for detection of FLAG. Background staining was <3%, whereas FLAG-transduced cells were 15–20% positive. An increase in appropriate transcript, A1 or Bcl-X_L, following transfection and selection in G418 was documented by RT-PCR (not shown). Levels of A1, normalized for β-actin, were consistently 1.5–2-fold greater than for Bcl-X_L in these cells (summary of four experiments).

As shown in Figure 3, both A1 and Bcl-X_L transduced MVECs, but not cells transduced with neo^R alone, were completely protected against the apoptotic effects of an 18-hr exposure to TNF-α in the presence of actinomycin D. Thus, despite probable variations in expression of A1 and Bcl-X_L transgenes in individual cells, levels of either molecule were sufficient to completely protect against TNF-α. The susceptibility of these cells to TTP plasma-mediated cell injury was then investigated. Bcl-X_L overexpression resulted in a 70.1% (mean of four experiments) decrease in A_0 values, when control (neo^R) and Bcl-X_L transduced ECs exposed to plasma from an HIV− individual with TTP were compared (Fig. 3). In the same system, A1 overexpression led to a mean 44% decrease in A_0 values (Fig. 3). This consistent difference was noted despite the fact that higher mean levels of A1 than Bcl-X_L expression were consistently obtained, and A1 mediated an equivalent or greater suppression of TNF-α mediated apoptosis when compared with Bcl-X_L.

DISCUSSION

EC damage is a fundamental event in TTP and sporadic HUS [3,6]. We investigated protease-based pathways for TTP/HUS plasma-mediated apoptotic injury of microvascular EC. We found that TTP/sporadic HUS plasmas up-regulated the cysteine protease caspase 1 and caspase 3, and tetrapeptide inhibitors of these enzymes suppressed plasma-mediated apoptosis. This inhibition occurred despite the fact that new protein synthesis was not required to obtain plasma-mediated cell death, supporting the concept of caspase activation via disruption of preformed complexes [9].

These findings parallel other features of our EC injury model for TTP. We had initially noted the ability of acute

TABLE III. Effect of Protease Inhibitors on TTP Plasma-Mediated MVEC Apoptosis*

Experiment	EC source	Plasma (1:100)	Inhibitor	[μM]	Apoptosis assessment	
					A_0 peak %	(%) Inhibition
1	MVEC-1	Control	—	—	0	—
	MVEV-1	TTP-8	—	—	30.4	—
		TTP-8	ATA	0.01	5.4	82.9
2	MVEC-1	Control	—	—	2.1	—
	MVEC-1	TTP-8	—	—	52.5	—
	MVEC-1	TTP-8	YVAD-H	2	30.4	42.1
	MVEC-1	TTP-8	YVAD-H	5	20.1	61.7
	MVEC-1	TTP-8	YVAD-H	10	0	100.
	MVEC-1	TTP-8	DEVD-H	100	0	100.
3	TEC	Control	—	—	0	—
	TEC	TTP-12	—	—	7.5	—
	TEC	TTP-12	YVAD-H	2	13.3	0
	TEC	TTP-12	YVAD-H	5	0	100.
	TEC	TTP-12	YVAD-CMK	2	3.8	49.3
	TEC	TTP-12	Okadaic acid	0.1	19.4	0
	TEC	TTP-12	Okadaic acid	0.5	0	100.
4	TEC	Control	—	—	0	—
	TEC	TTP-8	—	—	6.7	—
	TEC	TTP-8	YVAD-H	10	0	100.
	TEC	TTP-8	DEVD-H	100	0	100.
	TEC	TTP-8	Okadaic acid	0.5	0	100.

*TTP, thrombotic thrombocytopenic purpura; MVEC, microvascular endothelial cell.

but not remission TTP patient plasmas to induce Fas in MVECs susceptible to apoptosis in vitro [3–5], lineages which reflect the in vivo organ distribution of microthrombi in TTP/sporadic HUS. In turn, cross-linking of Fas results in sequential activation of caspases 1 and 3 [28–30]. However, Fas/Fas ligand interactions do not induce EC apoptosis [31], and anti-Fas mAbs had a minor impact on TTP plasma-mediated apoptosis of our microvascular ECs [3]. Fas up-regulation may thus simply represent a marker for plasma-mediated EC activation, consistent with morphologic evidence for such activation in vessel biopsies from TTP/sporadic HUS patients [6].

Our study demonstrates that, regardless of the identity of the plasma trigger(s) to EC apoptosis, the effector mechanism appears to be caspase-dependent. Early work with circulating factors in TTP suggested a prominent role for proteases in the thrombotic microangiopathies. A platelet aggregating cysteine protease found in acute TTP plasmas resembles a lysosomal cathepsin, and is thought to be released by injured ECs [32]. In addition, levels of a calcium-dependent cysteine protease (calpain) capable of cleaving ultra-high molecular weight von Willebrand factor (vWF) multimers correlate with TTP disease activity [33]. Finally, plasma metalloproteinases, released in the high shear stress environment of the arterial microcirculation, have been noted in TTP plasma [34]. The latter may be relevant to the marked increase in relative risk for TTP among HIV infected individuals [3], as expression of monocyte metalloproteinases is augmented by a secreted HIV regulatory gene product, Tat [35], and HIV protease can cleave Bcl-2 [9].

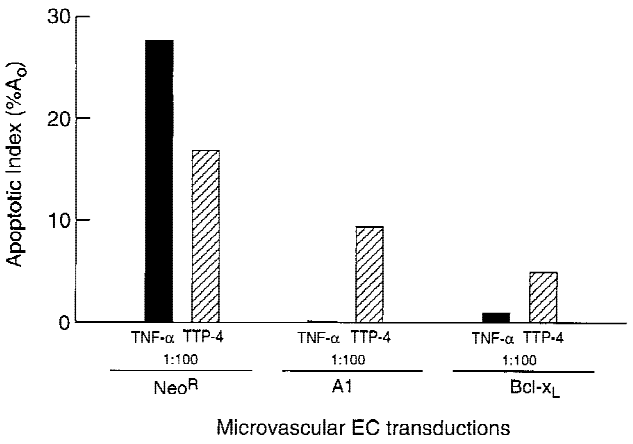

Fig. 3. Effect of Bcl-2 homolog A1 and Bcl-X_L overexpression on TTP plasma-mediated MVEC apoptosis. MVECs of dermal (MCEV-1) and cerebral (HMVEC-B) origin were transduced with genes for either resistance to neomycin alone (neo^R), or neo^R plus A1 or Bcl-X_L, then expanded in the presence of neomycin. Neomycin was removed from the cultures for 48 hr prior to testing of a particular EC population. ECs were exposed to normal plasma (1:100 dilution), PBS, TNF-α [0.05 ng/ml recombinant material, performed in the presence of actinomycin D (1 μg/ml)], or TTP-4 plasma (1:100 dilution). PI staining and DNA histogram analysis with calculation of areas under the A_0 peaks were performed 18 hr later. Results from experiments with dermal and cerebral ECs were pooled. There was no difference in background between PBS and normal plasma controls, and background A_0 values using either were subtracted from all samples. Standard deviations of <20% were seen for all assays.

Manipulation of apoptosis may lead to a new modality for the treatment of TTP/sporadic HUS. In general, apoptosis may be blocked at four discrete levels, reflecting a hierarchy of specificity, toxicity, and efficiency: interception of the apoptotic stimulus; functional antagonism of the apoptotic trigger at the receptor/ligand stage; interference with signal transduction cascades; and block of catabolic enzymes, including caspases, other proteases, and endonucleases, which participate in cell suicide [36]. Most nonspecific cysteine protease inhibitors, including okadaic acid and ATA, are highly cytotoxic [13]. Use of more specific protease inhibitors, based on caspase 1 and 3 substrates YVAD and DEVD, respectively, is limited by membrane permeability [13]. High extracellular concentrations can impede caspase-based apoptosis in vivo [26]. However, the incomplete specificity of these inhibitors, their short half-lives in plasma, and the possibility that noncaspase based pathways may be involved in TTP/sporadic HUS-associated cell death must be considered.

We also investigated whether physiologic inhibitors of apoptosis could suppress TTP plasma-mediated apoptosis. At least four proteins of the Bcl-2 family, Bcl-2, Bcl-X_L, A1, and Mcl-1 suppress apoptosis, whereas four others (Bax, Bcl-X_S, Bad, and Bak) promote it [37]. Cellular location and structural studies suggest mitochondrial membrane transport functions for these molecules [38]. Mitochondrial swelling is a cardinal feature of apoptotic cells [3,39], and is prominent in MVECs of involved tissues from TTP patients [6]. In isolated mitochondria, opening of permeability transition pores is accompanied by colloidosmotic swelling [39]. Failure to block release of mitochondrial proteins into the cytosol activates cysteine proteases or caspases that are the terminal effectors of apoptosis. Overexpression of Bcl-2 and Bcl-X_L can inhibit this permeability transition [10,38–41].

Slowing of entry of G_0 cells into the cell cycle, another effect of Bcl-2 and related molecules [38], could provide additional protection against cell death. Proliferating cells are more vulnerable to apoptotic stimuli; forcing cell cycle re-entry in differentiated cells is a classic method of inducing apoptosis [38]. Indeed, prior to the loss of ECs from involved vessels in TTP/sporadic HUS in vivo, intense EC activation and proliferation, occasionally occluding vessel lumina, is typical [4,6].

The relevance of the greater magnitude of suppressive effect of Bcl-X_L vs. A1 overexpression on TTP plasma vs. TNF-α-mediated apoptosis is unclear. However, it is known that Fas-associated cell death is associated with cleavage of Bcl-2, whereas Bcl-X_L is much less susceptible to this pathway [42], and chemotherapy-associated apoptosis is differentially affected by Bcl-2 and Bcl-X_L [43].

Options for TTP/HUS patients who do not respond to plasma exchange are limited. Splenectomy is of uncertain value [44]. Vincristine may be effective in some refractory cases, but its mechanism of action is unknown [44]. It disrupts tubulin, and such microtubule active agents inactivate Bcl-2 via hyperphosphorylation, suppressing the closing of the mitochondrial permeability transition pores [10]. Vincristine-mediated inhibition of A1 or Bcl-X_L might therefore be expected to exacerbate, not mitigate, apoptosis. However, homologous phosphorylation sites exist for the Bcl-2-like apoptosis-promoting gene products [10], and their inactivation would be of benefit. Interactions among pro- and anti-apoptotic physiologic MVEC factors such as these, their regulation by plasma triggers to EC apoptosis, and the involvement of the caspase cysteine proteases may aid in defining mechanisms of action for existing treatments for the thrombotic microangiopathies, as well as the nature of the apoptotic stimuli, and clues to their control.

ACKNOWLEDGMENTS

We thank George Lam for technical support, and Dr. Joel Moake, Baylor College of Medicine (Houston, TX), and Drs. Chis Krispas and Chau Dang, Cornell University Medical College, for providing patient plasma samples.

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